

## A RAPID PROCEDURE FOR PURIFYING A RESTRICTION ENDONUCLEASE FROM *THERMUS THERMOPHILUS* (*Tth* I)

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### 1. Introduction

Restriction enzymes have proved to be a powerful tool for mapping genomes [1] and developing molecular cloning and DNA sequencing techniques [2]. A few restriction enzymes have been isolated from thermophilic bacteria [3], showing a high thermostability and resistance to protein denaturing agents. These properties could be useful to study DNA structure at higher temperatures. Looking for a stable enzyme with a new recognition sequence, we have isolated *Tth* I, an enzyme from the extreme thermophile *Thermus thermophilus* HB8 [4]. This thermostable enzyme turned out to be an isoschizomer of *Taq* I, which recognizes the sequence 5'-TCGA-3' [5]. *Thermus thermophilus* is a very convenient source for purifying this enzyme since this bacterium does not produce the pigmented 'slime' described in *Thermus aquaticus* [6], which interferes with the purification of *Taq* I [5,7] and other enzymes [8,9]. Furthermore, *Tth* I was readily released by osmotic shock, which allowed us to develop a simplified, rapid two-step procedure to obtain an enzyme preparation free of contaminating nucleases. We report here the purification method and some of the properties of *Tth* I.

### 2. Materials and methods

#### 2.1. Source of materials

*Thermus thermophilus* HB8 strain was obtained from American Type Culture Collection (ATCC 27634). Cells were grown at 75°C with vigorous shaking and were harvested by centrifugation before the end of the log phase of growth. The culture media contained 4 g yeast extract, 6 g polypeptone and 3 g NaCl per liter. Agarose (1%) (Seakem) vertical slab gels were

run in 50 mM Tris-HCl, 40 mM sodium acetate and 1 mM EDTA (pH 8.0). To analyze small DNA fragments, acrylamide (Bio-Rad) gels were run in 90 mM Tris-HCl, 70 mM boric acid and 2.5 mM EDTA (pH 8.3). Gels were stained with ethidium bromide (1 µg/ml) and photographed under ultraviolet light. *Taq* I restriction enzyme was obtained from BRL.

#### 2.2. Preparation of DNAs

λ DNA was prepared from λ-lysogenic *E. coli* strain 594λcI857Sam7 (generously provided by H. Murialdo) as in [10]. PM2 phage DNA, *Col* E1 and pBR322 plasmid DNAs were prepared using standard procedures.

#### 2.3. Assay for restriction enzymes

Digestions with either *Tth* I or *Taq* I were carried out in 20 mM Tris-HCl (pH 8.0), 5 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol and 50 mM NaCl. DNA (~1 µg) dissolved in 10 mM Tris-HCl (pH 8.0). EDTA (1 mM) was added to 20 µl final vol. The samples were incubated for 60 min at 60°C and the reactions were halted with 10 µl solution containing 20% glycerol, 1% SDS, 50 mM EDTA and 0.05% bromophenol blue.

### 3. Results and discussion

#### 3.1. Purification steps

The isolation procedure involves osmotic shock treatment [11]. Bacterial cells (10 g) were washed twice with 200 ml of a buffer containing 10 mM Tris-HCl (pH 7.8) and 30 mM NaCl, resuspended in 200 ml 33 mM Tris-HCl buffer (pH 7.8) containing 3 mM EDTA and 20% sucrose and stirred at room temperature for 20 min. After centrifugation at 16 000 × g for 5 min, cells were resuspended in

150 ml 0.5 mM  $\text{MgCl}_2$ , stirred at room temperature for 10 min and centrifuged at  $16\,000 \times g$  for 5 min. The supernatant was made 20 mM Tris-HCl (pH 7.5), 4 mM 2-mercaptoethanol, 1 mM EDTA and 20% glycerol (buffer A) and applied to a DEAE-cellulose column ( $1 \times 15$  cm). The enzyme was eluted with a 400 ml linear gradient from 0–0.5 M NaCl in buffer A. The active fractions, eluting between 0.13–0.18 M NaCl were loaded onto a 5 ml phosphocellulose column pre-equilibrated with buffer A. The enzyme was eluted stepwise using 6 ml of buffer A containing 0.2, 0.3, 0.4, 0.5 and 0.6 M NaCl. The activity eluted at 0.6 M NaCl was concentrated by dialysis against 30% polyethylene glycol dissolved in buffer A. This final fraction was 0.5 ml and 0.65 mg protein/ml. At 2  $\mu\text{g}$ , this enzyme preparation produced complete digestion of 1  $\mu\text{g}$   $\lambda$  DNA in 30 min at  $60^\circ\text{C}$ . If the incubation time was extended for 12 h, the same sharp band pattern is observed in polyacrylamide gel electrophoresis. *Tth* I restriction enzyme can thus be obtained in 1 day free of contaminating nucleases. Since *Tth* I is released by osmotic shock, this enzyme is most probably located in the periplasmic space [12].

### 3.2. Properties of *Tth* I restriction enzyme

Searching for the recognition sequence of *Tth* I, we found that the pattern of restriction fragments produced after digestion with various DNAs was identical to that obtained with the *Taq* I enzyme (fig.1). Therefore, *Tth* I and *Taq* I endonucleases are isoschizomers.

The effect of incubation temperature and temperature stability of *Tth* I are shown in fig.2. Optimal incubation temperature is  $60^\circ\text{C}$ , showing very little activity at  $37^\circ\text{C}$  and incomplete digestion at  $50^\circ\text{C}$  or  $70^\circ\text{C}$ . Figure 2 also shows the thermostability of *Tth* I at  $80^\circ\text{C}$ . The enzyme was pre-incubated at this temperature for different time intervals and then assayed under standard conditions. A 10 min pre-incubation period does not affect *Tth* I activity, although after 1 h at  $80^\circ\text{C}$  the enzyme is partially inactivated.

*Tth* I is neither affected by NaCl up to 0.3 M, nor by changes in pH over 7.5–8.5. In contrast to other restriction enzymes [13],  $\text{MnCl}_2$  at 1.5 mM can replace efficiently  $\text{MgCl}_2$  without any change in enzyme specificity.

We have recently isolated a plasmid from *Thermus thermophilus* and are currently investigating if *Tth* I is a plasmid-coded enzyme.

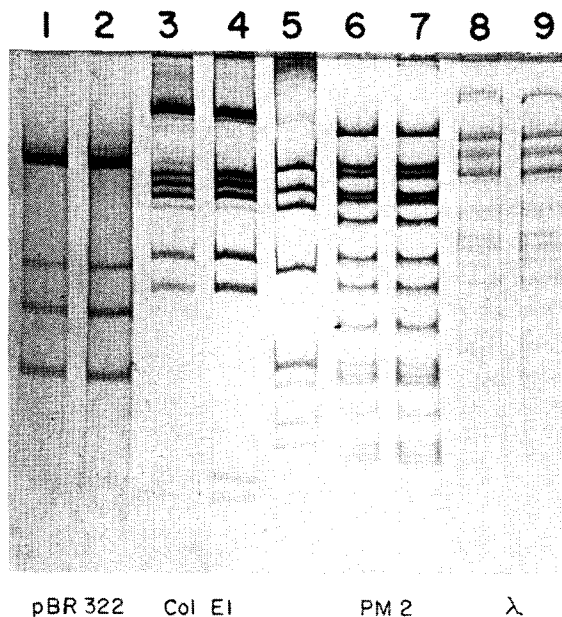


Fig.1. Digestion pattern of various DNAs with *Tth* I and *Taq* I. The DNA fragments produced were analyzed in a 7% acrylamide gel. Lanes 1,3,6,8: *Tth* I digestion. Lanes 2,4,7,9: *Taq* I digestion. Lane 5 contains molecular weight standards obtained by treatment of  $\phi\text{Xr}173$  DNA with *Hae* III restriction enzyme.

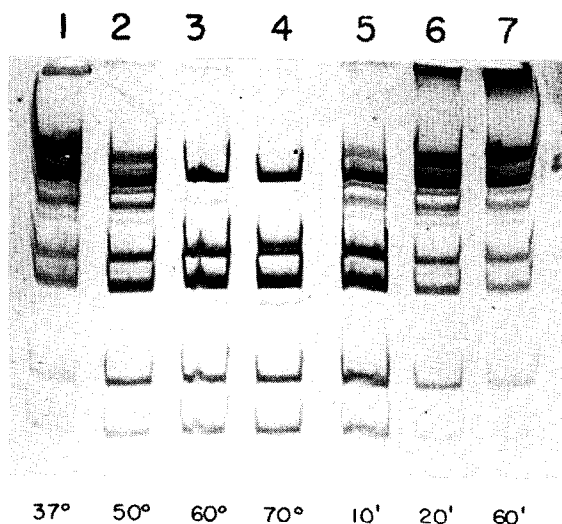


Fig.2. Effect of *Tth* I on *Col* EI DNA under different experimental conditions. The DNA fragments obtained were separated in a 5% acrylamide gel. Lanes 1–4: assays carried out at the temperatures indicated. Lanes 5–7: *Tth* I pre-incubated at  $80^\circ\text{C}$  for the indicated time intervals before assaying under standard conditions (see section 2).

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